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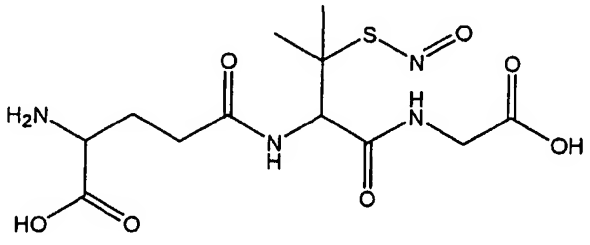
(54) Title: GLYCINE, N-[N-L-GAMMA-GLUTAMYL-3-(NITROSOTHIO)-L-VALYL] AND USE THEREOF



(I)

(57) Abstract: The present invention relates to glycine, N-[N-L-gamma-glutamyl-3-(nitrosothio)-L-valyl] having the following formula (I) *(see formula I in paper form)* and pharmaceutically acceptable salts thereof as well as pharmaceutical compositions comprising one or more of these compounds, methods for the treatment and prevention of diseases comprising administration of an effective amount of one or more of these compounds and the use of one or more or these compounds for the manufacture of a pharmaceutical composition for the treatment and prevention of diseases.

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Glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] and use thereof

Field of the invention

The present invention relates to glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] and pharmaceutically acceptable salts thereof as well as to pharmaceutical compositions comprising glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl], methods for the treatment and prevention of diseases comprising administration of an effective amount of glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] and pharmaceutically acceptable salts thereof and the use of glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] and pharmaceutically acceptable salts thereof for the manufacture of a pharmaceutical composition for the treatment and prevention of diseases.

Background of the invention

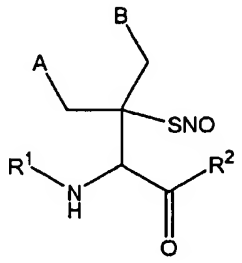
It is known that compounds capable of releasing nitric oxide (NO) in vivo exhibit various types of activity, for example a vasodilating activity and/or a platelet aggregation inhibiting activity, in the cardiovascular system. However, it is also well known that many of these compounds are highly unstable.

A certain class of compounds capable of releasing NO in vivo is represented by the so-called S-nitrosothiols.

For example, S-nitrosoglutathione (GSNO) and related compounds can exert various pharmaceutically relevant activities on the cardiovascular system, and are potentially useful in the treatment or prevention of a number of different diseases and medical conditions.

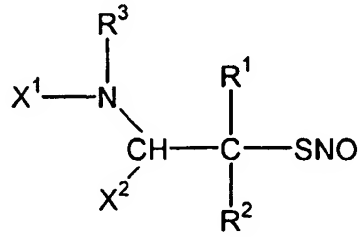
WO 95/07691 describes the use of nitric oxide donors such as S-nitrosoglutathione and S-nitrosopenicillamine for the prevention of thrombus formation on damaged vascular surfaces.

WO 00/44714 relates to a series of compounds given by the following formula:



in which A and B are phenyl groups or together form the rest $-CH_2-Q-CH_2-$ constituting a ring of six units in which Q represents an atom of oxygen, of sulphur, or a group $N-R^3$, in which R^3 is hydrogen or an alkyl group C_1-C_4; R^1 is an acyl rest, which may be an aliphatic acyl group C_1-C_5, or R^1 can be a rest of glutamic acid bound via its non-amino acid carboxyl; R^2 is a hydroxyl group or a glycine rest bound via a peptide bond; with the proviso that if R^1 is an aliphatic acyl rest then R^2 is a hydroxyl group, and if R^1 is a rest of glutamic acid then R^2 is a glycine rest; as well as their use in the treatment of circulatory dysfunctions.

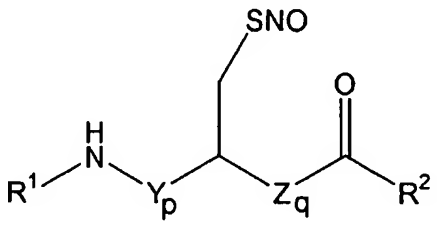
EP 412 699 describes S-nitrosothiols which correspond to the following formula:



and their use as therapeutic agents against cardiovascular disease, in particular as anti-hypertension agents and as

agents for the treatment of angina pectoris. However, the number of possible compounds within said formula is enormous and there is no indication that these compounds have any effect on the aggregation of platelets.

WO 96/16645 relates to certain S-nitrosothiol compounds having the following formula:



and their use as pharmaceuticals for the treatment of thrombic conditions involving platelets, for example, as anti-thrombotic agents and agents for the treatment and prevention of restenosis. However, the number of possible compounds within said formula is enormous.

In Nitric Oxide (1998), vol. 2(3), 193-202, several S-nitrosated dipeptides were synthesized, including SNAP-gly (S-nitroso-N-acetyl-D,L-penicillamine-glycine), and tested for their activity as vasodilators.

Thus, some S-nitrosothiols and related compounds have an inhibitory effect on platelet aggregation, whereas other related compounds are known to have an effect on vasodilatation, i.e. relax vascular smooth muscles. Some may have an effect on both.

Summary of the invention

In light of these two different demonstrated physiological effects of this class of compounds, it would be advantageous to have compounds that are more selective for one of these two effects.

An object of the present invention is to provide a compound that has an improved ability to inhibit platelet aggregation with respect to their ability to relax vascular smooth muscles.

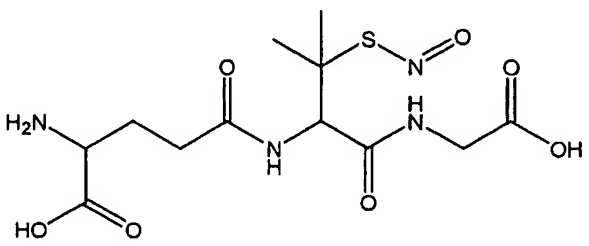
A further object of the invention is to provide pharmaceutical compositions that have an improved ability to inhibit platelet aggregation with respect to their ability to relax vascular smooth muscles.

A further object of the present invention is to provide methods for the prevention or treatment of various diseases or medical conditions that can benefit from the improved ability of a compound having an improved ability to inhibit platelet aggregation with respect to its ability to relax vascular smooth muscles.

Another object of the present invention is to provide for the use of a compound having an improved ability to inhibit platelet aggregation with respect to its ability to relax vascular smooth muscles in the manufacture of a pharmaceutical composition for the treatment or prevention of a disease or medical condition.

Detailed description of the invention

The above objects are solved according to the invention by providing a compound according to the following formula (I)

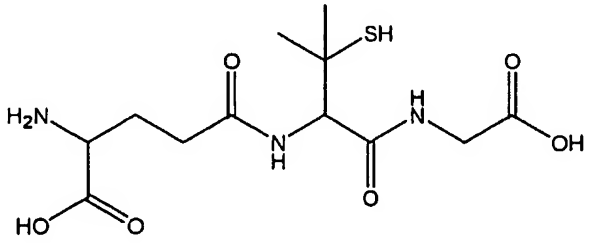


(I)

or a pharmaceutically acceptable salt thereof.

Compounds of the present invention also include pharmaceutically acceptable salts of the compound of formula (I). These salts include those with alkali metals, alkaline earth metals and ammonium such as the sodium, potassium, lithium, calcium, magnesium, barium and ammonium salts. These salts also include acid addition salts. Suitable acids for the formation of acid addition salts are hydrochloric, hydrobromic, sulfuric, acetic, oxalic, valeric, oleic, lauric, boric, benzoic, lactic, phosphoric, toluene sulfonic, citric, maleic, fumaric, succinic, tartric, sulfonic, glycolic, ascorbic, benzenesulfonic, nitric, trifluoroacetic and the like acids.
A preferred pharmaceutically acceptable salt of the compound of formula (I) is the hydrochloride salt.

The compound of the formula (I) can be produced by nitrosation of the compound represented by the formula (II).



(II)

Reagents generally used for nitrosation of the compound (II) include nitrogen monoxide, nitrogen dioxide, dinitrogen tetraoxide, nitrosyl chloride, nitrous acid, tertbutyl nitrite, and ethyl nitrite, but the reagents are not limited to these, and any reagent that can usually be used for nitrosation may be used.

Reagents generally used for acidification include citric acid, and hydracidic acids, and any reagent that can usually be used for acidification may be used. It is usually used the nitrous acid generated in situ by addition of a hydrochloric acid solution to a sodium nitrite solution.

Reagents generally used for inhibition of decomposition of the compound (I) added at the end of the reaction include EDTA, and any reagent that can usually be used as ion chelator.

The reaction may be conducted without any solvent or in a solvent. Any solvent may be used as far as it does not inhibit nitrosation, including water, alcohols (e.g. methanol, ethanol, propanol, butanol, tert-butanol), petroleum-composing solvents (e.g. n-hexane, n-pentane, n-heptane), aromatic solvents (e.g. benzene, toluene, pyridine), ethers (e.g. ethyl ether, tetrahydrofuran, dioxane, isopropyl ether), amides (e.g. N,N-dimethyl-formamide, N-N-dimethylacetamide), esters (e.g. methyl acetate, ethyl acetate, butyl acetate), halogenated hydrocarbons (e.g. dichloromethane, chloroform, dichloroethane, carbon tetrachloride), and dimethylsulfoxide.

The reaction can be conducted at temperatures from -30°C to 150°C, but it is preferably conducted at a lower temperature (-5°C to 30°C). For one mole of the compound (II), preferably 1 to 5 moles of the nitrosating reagent is used. The reaction time varies from 1 minute to 6 hours, preferably as short as 1 minute to 30 minutes.

Furthermore, the present invention provides a pharmaceutical composition comprising a compound according to formula (I) or a pharmaceutically acceptable salt thereof, optionally together with one or more other active ingredients, and/or one or more pharmaceutically acceptable carriers.

For example, the pharmaceutical composition of the invention can also comprise thrombolytic agents as active ingredients. Preferred thrombolytic agents are plasminogen activator, urokinase, streptokinase, anistreplase, scuPA.

The pharmaceutical composition of the invention can also comprise anticoagulant agents. Preferred anticoagulant agents are heparin, coumarin and pentosan polysulfate.

The pharmaceutical composition of the invention can also comprise antithrombotic agents. Preferred antithrombotic agents are aspirin, dipyridamole, ticlopidine, clopidrogel, triflusal, pentosan polysulfate and abciximab.

In addition, the pharmaceutical composition according to the invention can also comprise an immunoglobulin or fragment thereof having a specificity for glycoprotein IIb/IIIa as described in the patent US 5,976,532 as an active ingredient.

Mixtures of two or more of the above active ingredients are included within the meaning of the pharmaceutical compositions of the present invention.

Pharmaceutical compositions according to the invention can be formulated along with customary pharmaceutical carriers, vehicles and other additives into tablets, powders, fine granules, capsules, pills, liquid preparations, injections, suppositories, ointments, cataplasms and the like and can be administered in an oral, parenteral, rectal, transnasal, topical, transdermal, vaginal and sublingual manner.

The clinical dose of the compound of formula (I) or a pharmaceutically acceptable salt thereof may be suitably determined, depending on the specific mode of administration, the specific symptoms, the body weight, the age and the sex of the patient to which they are administered, but is generally from 0.5 mg to 500 mg/adult/day. This dose may be administered to the patient as one daily dose or may be divided into several doses during the day.

As a solid pharmaceutical composition for administration of the compound of formula (I) or a pharmaceutically acceptable salt thereof, tablets, powders, granulates can be employed.

Solid compositions of this type comprise one or more active substances along with at least one inert diluent such as lactose, mannitol, glucose, hydroxypropyl cellulose, microcrystalline cellulose, starch, polyvinyl pyrrolidone, meta-silicic acid, and magnesium aluminate. In a customary manner, the pharmaceutical composition of the present invention may contain any other additives aside from those mentioned above, for example, a lubricants such as magnesium stearate, a disintegrator such as calcium cellulose glycolate, a stabilizer such as lactose and a solubilizer or dissolution promoter such as glutamic acid or aspartic acid. If desired, the tablets or pills may be coated with a film of gastric or enteric resistant substances such as sucrose, gelatin, hydroxypropyl cellulose, hydroxypropylmethycellulose phthalate, etc.

A liquid pharmaceutical composition according to the invention for administration of the compound of formula (I) or a pharmaceutically acceptable salt thereof includes, for example, pharmaceutically acceptable emulsions, solutions, suspensions, syrups, elixirs, and the like, which comprise customary inactive diluents such as distilled water and ethyl alcohol, buffers and physiological saline or salt solutions. In addition, these compositions may further contain aids for pharmaceutical formulation such as solubilizers, dissolution promoters, wetting promoters, suspension promoters and sweeteners, flavorings, aromas, colorants and preservatives.

The pharmaceutical composition of the present invention encompasses not only liquid compositions, but also lyophilized preparations for reconstitution in water, buffers or physiological saline.

Injections containing the compound of formula (I) or a pharmaceutically acceptable salt thereof includes, for example, aqueous or non-aqueous solutions, suspensions and emulsions, all of them germ-free. The diluent for the aqueous solutions and suspensions includes, for example, distilled

water, buffers, physiological saline and salt solutions. The diluent for non-aqueous solutions and suspensions includes, for example, propylene glycol, polyethylene glycol, vegetable oils such as olive oil, alcohols such as ethyl alcohol, Polysolvate 80®. These compositions may further contain additives such as isotonicating promoters, preservatives, wetting promoters, emulsifiers, dispersants, stabilizers, solubilizers, dissolution promoters, etc.

The compound of formula (I) or a pharmaceutically acceptable salt thereof may also be administered as a pharmaceutical composition suitable for topical administration in the form of, for example, creams, ointments, pomades, balsams, etc. or as a pharmaceutical composition suitable for transdermal administration in the form of, for example, patches or bandages.

The compound of formula (I) or a pharmaceutically acceptable salt thereof may also be administered as a pharmaceutical composition suitable for rectal administration in the form of a suppository.

Thus, the present invention provides for the use of a compound according to formula (I) or a pharmaceutically acceptable salt thereof, for the manufacture of a pharmaceutical composition for the prevention or treatment of a disease or a medical condition.

Moreover, the present invention provides a method for the treatment or prevention of a disease comprising the administration of an effective amount of a compound according to formula (I) or a pharmaceutically acceptable salt thereof to a patient in need thereof.

Preferably, the disease or medical condition is selected from the group consisting of thrombic disorders, stent-induced restenosis, platelet activation during extracorporeal circulation of the blood, preferably during surgery or in

hemodialysis, platelet aggregation, coagulation and neointimal formation.

The present invention is more closely illustrated by means of the following examples, but the invention should not be considered as being limited to the examples.

Examples

The compound of example 1 below is identified via Nuclear Magnetic Resonance spectroscopy of protons (^{1}H-NMR) and of carbon 13 (^{13}C-NMR), Heteronuclear Chemical Shift Correlation (HETCOR), and mass spectrometry (MS).

The Nuclear Magnetic Resonance spectra (^{1}H-NMR, ^{13}C-NMR and HETCOR) have been realized with a Varian Gemini 2000 apparatus.

In the ^{1}H-NMR spectra the working frequency and the solvent used for obtaining the spectrum are indicated. The position of the signals is indicated in δ (ppm), using the signal of the protons of the solvent as reference. The reference value for the deuterated dimethylsulfoxide (DMSO-d6) is 2.48 ppm. The number of protons corresponding to each signal measured by electronic integration and the type of signal is indicated using following abbreviations: s (singlet), d (doublet), t (triplet), bs (broad signal), and sc (complex signal).

In the ^{13}C-NMR spectra the working frequency and the solvent used for obtaining the spectrum are indicated. The position of the signals is indicated in δ (ppm), using the signal of the carbons of the solvent as reference. The reference value for the deuterated dimethylsulfoxide (DMSO-d6) is 38.5.

In the HETCOR spectra the solvent used for obtaining the spectrum are indicated. The position of the signals is indicated in δ (ppm) using the signal of the protons and

carbons of the solvent as reference. The number of protons and carbons corresponding to each cross peak and the type of signal are indicated. The abbreviations used to describe the type of signal are the same as used for the ^{1}H-NMR spectra.

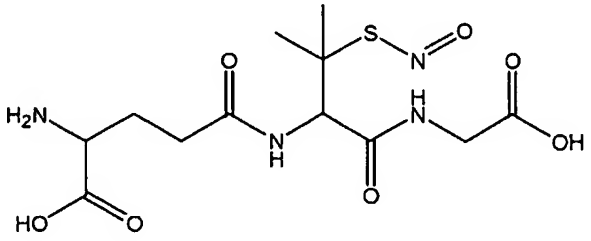
The overall purity of example 1 below has been determined by HPLC chromatography using a reverse phase and quantifying the compound by area percentage at 340 nm. The HPLC tests have been realized in a conventional HPLC apparatus with Diode Array Detector.

In the MS spectra the M+H ion is indicated as a molecular ion plus proton, and also the M+H-NO as a base ion.

The MS spectra have been realized with a conventional HPLC system coupled to a Micromass VG Quatro equipment using a positive electrospray as atmospheric interface.

Example 1: Synthesis of glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl]

To a solution of glycine, N-[N-L-γ-glutamyl-3-thio-L-valyl] (0.1 g, 0.299 mmol) in 1 ml 1 N hydrochloric acid, it was added dropwise in an ice bath 0.06 ml of sodium nitrite 5 M. After stirring for 30 minutes, 0.1 ml of sodium hydroxide 10 N and 0.05 ml of EDTA 0.002 M were added. The solution was evaporated in a freeze-dryer to obtain 156 mg of crude product . After that, 90 mg of crude product were dissolved in 0.8 ml methanol, filtrated and evaporated to yield glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] as a green solid (53 mg).



(I)

^{1}H-NMR (200 MHz, DMSO-d6): 1.72-1.94 (2H, sc, CH_2, C7); 1.92 (3H, s, CH_3); 2.04 (3H, s, CH_3); 2.22-2.38 (2H, sc, CH_2, C8); 3.29 (1H, t, J=9Hz, CH_{Glu}, C4); 3.68 (2H, sc, CH_2, C9); 5.22 (1H, d, J=15Hz, CH_{val}, C5), 8.70-8.85 (1H, bs).

^{13}C-NMR(50 MHz, DMSO-d6): 170.8 (CO), 170.3 (CO), 170.0 (CO), 167.4 (CO), 58.7 (C_{val}, C6), 58.0 (CH_{val}, C5), 52.1 (CH_{Glu}, C4), 40.6 (CH_2 Gly, C9), 30.3 (CH_2 Glu, C8), 26.0 (CH_2 Glu, C7), 26.0 (CH_3), 23.5 (CH_3).

HETCOR (DMSO-d6):
5.22 (1H, d, J=15Hz, CHval, C5) - 58.0 (CH_{val}, C5)
3.29 (1H, t, J=9Hz, CH_{Glu}, C4) - 52.1 (CH_{Glu}, C4)
3.68 (2H, sc, CH_2, C9) - 40.6 (CH_2 Gly, C9)
2.22-2.38 (2H, sc, CH_2, C8) - 30.3 (CH_2 Glu, C8)
1.72-1.94 (2H, sc, CH_2, C7) - 26.0 (CH_2 Glu, C7)
2.04 (3H, s, CH_3) - 26.0 (CH_3)
1.92 (3H, s, CH_3) - 23.5 (CH_3)

HPLC Purity: 95% (340 nm)

MS (Electrospray +):
(M+H)+ : 365
[(M+H)+]-NO: 335

Example 2: Vasodilating activity

The method used in the assay of this example is substantially the same as described in Furchgot, R.F. "Methods in nitric oxide research", Feelisch & Stamler eds., John Wiley & Sons, Chichester, England, pp 567-581; Trongvanichnam, K. et al., Jpn. J. Pharmacol. 1996, 71: 167-173; and Salas, E. et al., Eur. J. Pharmacol. 1994, 258: 47-55.

The compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl], was assayed in vitro to determine its vasorelaxant

activity in aorta isolated from rat. The compound was added to the incubation medium of aortic rings after inducing a contraction with a sub-maximum concentration of Norepinephrine (1 μM). The compound has been tested at different concentrations (0.001 to 10 mM) using aortic rings from 6 different animals. The results obtained are compared to those from the S-nitrosoglutathione (GSNO), which was used as reference product.

These results are shown in table 1 and are provided as EC_{50} (effective concentration 50), which is the concentration of the compound that produced a vasorelaxation that represents 50% of the tone obtained by Norepinephrine (1 μM).

Table 1. Vasodilatation

Product	EC_{50} (μM) (mean ± SE)
GSNO	1.18 ± 0.13
Glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl]	10.54 ± 1.69 (*)

(*) $p<0.001$

As can be seen from table 1, the vasodilator strength of the compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] was approximately 10-fold lower than that of the compound GSNO.

Example 3: Inhibition of platelet aggregation in washed platelets

The method used in the assays of this example is substantially the same as described in Loscalzo J. et al., "Methods in nitric oxide research", Feelisch & Stamler eds., John Wiley & Sons, Chichester, England, pp 583-591; Radomski, MW, et al., Br. J. Pharmacol. 1987, 92: 181-187; and Salas, E. et al., Hr. J. Pharmacol, 1994, 112: 1071-1076.

The compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] was assayed in vitro to determine its inhibitory activity of the platelet aggregation. The compound was added to preparations of human washed platelets (WP) before inducing the aggregation with a sub-maximum collagen concentration (1 μg/ml). The compound was tested at 4 different concentrations using platelets from 9 different donors. The results obtained are compared to those from GSNO that was used as reference product.

These results are shown in table 2 and are expressed as IC_{50} (inhibitory concentration 50), which is the concentration of the compound that produced a 50% inhibition of the aggregation induced by collagen (1 μg/ml).

Table 2. Inhibition of platelet aggregation in human washed platelets (WP).

Product	IC_{50} (μM) (mean ± SE)
GSNO	0.37 ± 0.04
Glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl]	0.29 ± 0.05 (*)

(*) $p<0.005$

As can be seen from table 2, the compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] has a higher potency as an inhibitor of platelet aggregation than the reference product, GSNO. Furthermore the IC_{50} of the compound for inhibiting platelet aggregation is approximately 36-fold lower than its EC_{50} for vasorelaxant activity previously described, whereas in the case of GSNO, it is only 3-fold lower.

Example 4: Disaggregation of platelets in platelet rich plasma

The method used in the assays is substantially the same as described in the references cited above.

The compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] was assayed in vitro to compare its activity on platelet disaggregation. The compound was added to the human platelet rich plasma (PRP) one minute after inducing the aggregation with a sub-maximum ADP concentration (2 μg/ml). The compound was tested using plasma from 9 different donors. The results obtained are compared to those from GSNO as reference product.

The results are shown in table 3 expressed as IC_{50}. The IC_{50} is the concentration of the compound that produced a reversion of 50% of the aggregation after the aggregation was generated by ADP stimulation.

Table 3. Reversion of platelet aggregation in PRP.

Product	IC_{50} (μg/ml) (mean ± SE)
GSNO	0.29 ± 0.02
Glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl]	0.19 ± 0.08 (*)

(*) $p<0.01$

As can be seen from table 3, the compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] is a potent inductor of platelet disaggregation, reverting the platelet aggregation when platelet aggregates have already been produced.

Example 5: Increase of the intra-platelet level of cGMP

The method used in the test is substantially the same as described in the references cited previously.

The compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] was assayed in vitro to evaluate its capacity to

increase the intra-platelet levels of cGMP in a preparation of washed human platelets. The compound was tested at 4 different concentrations using platelets from 5 different donors. The results obtained are compared to those from the GSNO (reference product) and with the basal values.

These results are shown in table 4 and are expressed as pmol/10^9 platelets.

Table 4. Increase in the intra-platelet level of cGMP.

Concentration (μM)	cGMP (pmol/10^9 platelets) (mean ± SE)	
	GSNO	Glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl]
1	24 ± 0.5	41.2 ± 0.5 (*)
0.3	6.6 ± 0.6	6.10 ± 2.0
0.1	1 ± 0.5	0.9 ± 0.10
0	1.33 ± 0.38	1.33 ± 0.38

(*) p>0.0001

As can be seen from table 4, the compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] increases the intra-platelet levels of cGMP in a similar manner as GSNO, except at concentration 1 μM. Levels of cGMP induced by this concentration are approximately two-fold higher than those induced by GSNO.

Example 6: Liberation of NO

The method used in the assays is substantially the same as described in Askew, SC. et al., Bioorg. Med. Chem. 1995, 3(1): 1-9; Gorren, ACF. et al., Archs. Biochem. Biophysics. 1996, 330 (2): 219-229; and Simonsen, G. et al., Eur. J. Physiol. 1998, 935 (6): 16-24.

The release of nitric oxide (NO) from the compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] was tested in vitro at a concentration of 10 μM in the following different tissues and mediums: isolated aortic rings from rats, isolated human platelets, platelet rich human plasma and a saline solution comprising 100 mM of $CuSO_4$. The NO released by the compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] was measured in the various samples 3 times, with a specific and selective sensor of NO (World Precision Instrument). The results obtained are compared to those from GSNO as reference product. The results are shown in table 5.

Table 5. NO release (μmol NO)

Medium	GSNO (10 μM) (mean ± SE)	Glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] (10 μM) (mean ± SE)
$CuSO_4$ Saline Solutions	2.5 ± 0.16	12.5 ± 2.52 (*)
Aortic rings	3 ± 0.05	3.5 ± 0.22
Platelet rich plasma	0.8 ± 0.002	1.0 ± 0.041
Washed Platelet	5 ± 0.12	30 ± 3.58 (**)

(*) $p<0.05$, (**) $p=0.01$

As can be seen from table 5, the compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] produces a liberation of NO that is considerably higher in quantity than GSNO in solution with $CuSO_4$ and washed platelets.

The examples demonstrate that the compound of the present invention has an improved ability to inhibit platelet aggregation with respect to its ability relax vascular smooth muscles as compared to GSNO.

Example 7: Inhibition of p-selectin, of the expression of glycoprotein IIb-IIIa activated complex PAC-1 and platelet microparticle formation.

The method used in these assays is substantially the same as described in Becker, R.C. et al., Coron. Artery. Dis. 1994;5:339-345 (p-selectin); Shattil, S.J. et al., Immunomethods 1993;1:53-63 (expression of glycoprotein IIb-IIIa activation complex9; and Miller, D.T. et al., Thromb. Haemost. 1987;58:484-486 (platelet microparticle formation).

1. P-selectin and GP IIb-IIIa expression

The flow cytometric analysis of platelets was carried out within 10 minutes of blood collection. Platelet rich plasma (PRP) was added to 0.1 mol/L phosphate buffer containing appropriately diluted antibody and agonist. The mixture was then incubated at room temperature for 10 minutes. Afterwards, samples were diluted and fixed for 35 minutes at room temperature with 2% paraformaldehyde and 0.1% glutaraldehyde and subsequently diluted in 0.1 mol/L phosphate buffer. Finally, the mean of the fluorescence bound to platelets was measured by cytometry (Coulter EPICS XL-MCL).

Translocation of the α-granule glycoprotein p-selectin to the plasmatic membrane of platelet activated with ADP was determined using one monoclonal antibody against CD62P conjugated with PE (Dako, Spain). Expression of GP IIb-IIIa complex of ADP-activated platelets was determined using one monoclonal antibody against the activated complex (PAC-1 Becton Dickinson, USA).

2. Formation of microparticles.

The characterization and quantification of microparticles formed during the activation of platelet with thrombin was performed by flow cytometry. This method revealed a unimodal

decrease in forward light scatter of the platelet suspension. The microparticles may be differentiated from platelets and aggregates by placing the cursor on the left forward light scatter axis at the boundary of the non-activated platelet distribution.

The results obtained are compared to those from GSNO used as reference product. The results are shown in table 6.

Table 6

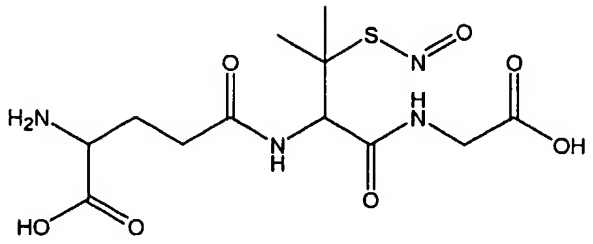
Compound	Inhibition of p-selectin expression (IC_{50}, µM) (mean ± SE)	Inhibition of PAC-1 expression (IC_{50}, µM) (mean ± SE)	% inhibition of platelet microparticle formation using 1 µM of the compound (mean ± SE)
GSNO	0.116 ± 0.02	0.276 ± 0.12	31.5 ± 7
Glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl]	0.095 ± 0.01	0.263 ± 0.079	42 ± 8

As can be seen from table 6, the compound glycine, N-[N-L-γ-glutamyl-3-(nitrosothio)-L-valyl] produces a considerable inhibition in the expression of p-selectin on the surface of platelets, the expression of the glycoprotein IIb-IIIa activated complex and the formation of platelet microparticles in comparison to GSNO.

The examples demonstrate that the compound of the present invention have an improved ability to act as anticoagulation agents and prevent neointimal formation as compared to GSNO.

Claims

1. A compound according to the following formula (I)



(I)

or a pharmaceutically acceptable salt thereof.

2. A compound according to claim 1, wherein the pharmaceutically acceptable salt is a hydrochloride salt.

3. A pharmaceutical composition comprising a compound according to claim 1 or 2 or a pharmaceutically acceptable salt and optionally a pharmaceutically acceptable carrier.

4. A pharmaceutical composition according to claim 3 further comprising a thrombolytic agent, preferably plasminogen activator, urokinase, streptokinase, anistreplase or scuPA.

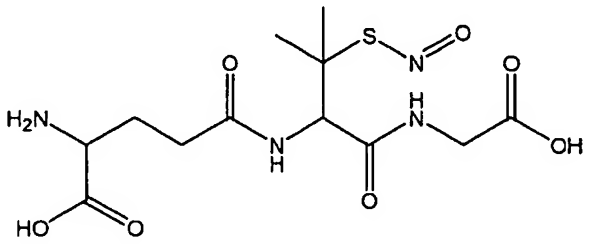
5. A pharmaceutical composition according to claim 3 or 4 further comprising an anticoagulant agent, preferably heparin, coumarin or pentosan polysulfate.

6. A pharmaceutical composition according to any of claims 3 to 5 further comprising an antithrombotic agent, preferably aspirin, dipyridamole, ticlopidine, clopidrogel, triflusal, pentosan polysulfate and abciximab.

7. A pharmaceutical composition according to any of claims 3 to 6 further comprising an immunoglobulin or fragment thereof having a specificity for glycoprotein IIb/IIIa.

8. A method of treatment or prevention of thrombic disorders, stent-induced restenosis, platelet activation during extracorporeal circulation of the blood, preferably during surgery or in hemodialysis, platelet aggregation and neointimal formation in a patient comprising administering an effective amount of a compound according to claim 1 or 2 or a pharmaceutical composition according to any of claims 3 to 7 to the patient.

9. Use of a compound according to the following formula (I)



(I)

or a pharmaceutically acceptable salt thereof, for the manufacture of a pharmaceutical composition according to any of claims 3 to 7 for the prevention or treatment of thrombic disorders, stent-induced restenosis, platelet activation during extracorporeal circulation of the blood, preferably during surgery or in hemodialysis, platelet aggregation and neointimal formation.